

THE FUNCTION OF sRNA* AS AMINO ACID ADAPTOR IN THE SYNTHESIS OF HEMOGLOBIN

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According to the adaptor hypothesis of Crick¹ and Hoagland,² the position of a particular amino acid in a polypeptide chain is determined, not by direct interaction between amino acid and template, but through the mediation of an sRNA molecule that functions as an adaptor. The hypothesis was tested by Chapeville *et al.*³ in a cell-free protein-synthesizing system from *E. coli*, using a synthetic polynucleotide as template. Cysteine attached to sRNA was converted to alanine, forming an Ala-sRNA^{CySH} hybrid (Fig. 1). The alanine was transferable into polypeptide in response to poly UG, which ordinarily stimulates the incorporation of cysteine but not of alanine. The present investigation shows that the result indicated by the use of the artificial messenger poly UG applies as well to the synthesis of hemoglobin, thus confirming the validity of the adaptor hypothesis in the synthesis of natural protein.

Materials and Methods.—*Preparation of C¹⁴ CySH-sRNA^{CySH} and of C¹⁴ Ala-sRNA^{Ala}:* sRNA from *E. coli* strain B was prepared by phenol extraction, stripped of amino acids, dialyzed, and lyophilized as described.⁴ It was charged with C¹⁴ cysteine, formed from C¹⁴ cystine (Nuclear Chicago Corp., DL + meso cystine-3-C¹⁴, 18.7 $\mu\text{C}/\mu\text{M}$) by the presence of β -mercaptoethanol, or with C¹⁴ alanine (New England Nuclear Corp., uniformly labeled, 71 $\mu\text{C}/\mu\text{M}$). The charging mixture⁴ contained, per ml (in micromoles, unless otherwise stated): Tris-HCl buffer, pH 7.2, 100; MgCl₂, 5; KCl, 5; β -mercaptoethanol, 5; C¹⁴ cysteine, 0.2 or C¹⁴ alanine, 0.02; a complementary mixture (i.e., cysteine or alanine omitted) of 19 nonradioactive amino acids, 0.2 each; ATP, 2.5; PEP, 20; PEP kinase, 30 μg ; and a dialyzed 100,000 $\times g$ supernatant of *E. coli* extract, 1.5 mg protein; sRNA, 20 mg. The aminoacyl-sRNA was recovered by phenol extraction, precipitated with ethanol, and dialyzed. It had a specific activity of 3,160 cpm per mg of sRNA in the case of cysteine, and 6,750 cpm per mg sRNA in the case of alanine. Two preparations of *E. coli* sRNA charged with a mixture of 19 nonradioactive amino acids (omitting cysteine or alanine) were also made.

Preparation of the hybrid C¹⁴Ala-sRNA^{CySH}: Stripped *E. coli* sRNA was charged with C¹⁴ cysteine in the presence of C¹² alanine (the remaining amino acids being omitted). The yield was 29 mg of sRNA with a specific activity of 4,000 cpm per mg of sRNA. It was reduced with Raney nickel as described by Chapeville *et al.*,³ and 19.5 mg of sRNA was obtained with a specific activity of 3,050 cpm per mg. The extent of the reduction was determined on an aliquot of the sample as described previously.³ Of the radioactivity bound to sRNA, 50% was identified as C¹⁴ alanine and 50% as C¹⁴ cysteine.

Cell-free incorporation into hemoglobin: Ribosomes from rabbit reticulocytes were prepared as described by Schweet *et al.*⁵ They were incubated with the appropriate aminoacyl-sRNA in a mixture containing the following (in micromoles per ml, unless otherwise noted): Tris-HCl, pH 7.8, 100; KCl, 50; MgCl₂, 5; GTP, 0.2; PEP, 7; PEP kinase, 50 μg ; GSH, 8; a balanced mixture of all 20 amino acids in the concentrations specified by Borsook *et al.*,⁶ but modified to contain 0.5 μmoles cysteine and 1.0 μmole alanine; 20 mg of sRNA charged with a complementary mixture of 19 nonradioactive amino acids; ribosomes and C¹⁴ aminoacyl-sRNA, as indicated in the legends of Figures 3, 4, and 5.

After incubation for 60 min at 37°C, the reaction mixture was chilled and 20 mg of rabbit hemoglobin was added as carrier. The ribosomes were removed by centrifugation at 100,000 $\times g$ for one hr. Ribonuclease (3 μg per ml) was added to the supernatant and the mixture kept at room temperature for one hr to degrade the sRNA. Globin was then prepared by the acid-acetone method.⁷ The globin was washed with acetone at -20°C and dissolved in 10 ml of the

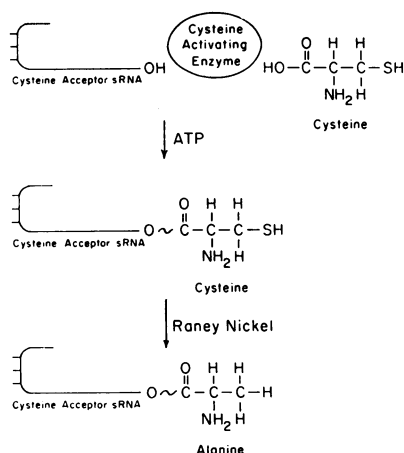


FIG. 1.—Plan of experiment. Cysteine is attached to its normal acceptor sRNA by the cysteine-activating enzyme. Raney nickel converts the cysteine, while still attached, to alanine, producing the hybrid Ala-sRNA^{CySH}. The coding properties of the hybrid molecule are then investigated. (After Chapeville *et al.*³)

Chicago) with a background of 1.5 cpm. Each sample was counted for at least 300 counts.

The insoluble cores remaining after trypsin digestion of the α -chains were eluted from the paper with 0.1 *N* HCl. After digestion with chymotrypsin in the presence of mercaptoethanol, the resulting peptides were separated by paper electrophoresis and chromatography,⁸ eluted, and counted.

After counting, the tryptic peptides were oxidized with performic acid and hydrolyzed in 6 *N* HCl for 36 hr at 105°C. The HCl was evaporated, alanine and cysteic acid were added as carriers, and the amino acids were separated at pH 1.9 by paper electrophoresis.¹⁰ The spots were made visible by staining with ninhydrin and those corresponding to alanine and cysteic acid were cut out, eluted, and counted as described above. The chymotryptic peptides, after counting, were oxidized with performic acid, hydrolyzed, and their amino acid composition determined semiquantitatively by paper electrophoresis in two stages at pH 6.4 and pH 1.9 as described by Naughton and Hagopian.¹⁰ The peptides were identified by comparing their amino acid compositions with the data of Diamond and Braunitzer.¹¹ The areas corresponding to cysteic acid and alanine were then cut out, eluted, and counted as described above.

Results.—Transfer of radioactive amino acids into the α - and β -chains of hemoglobin: Amino acids were transferred from *E. coli* aminoacyl-sRNA into hemoglobin in a rabbit reticulocyte ribosome system.⁴ Three transfer experiments were performed in which the sRNA was charged with C¹⁴Ala-sRNA^{Ala} or with C¹⁴CySH-sRNA^{CySH} or with Raney nickel-treated C¹⁴CySH-sRNA^{CySH}. As shown in Figure 2, the transfer reaction was almost complete within 10 min. The rate of incorporation from Raney nickel-treated CySH-sRNA^{CySH} was lower, but the final per cent of input transferred was the same.

The hemoglobin was isolated after the addition of carrier, converted into globin, and separated into α - and β -chains (see *Materials and Methods*). Consistent with earlier results obtained with leucine showing that both chains are synthesized in the cell-free system,⁴ alanine was transferred from C¹⁴Ala-sRNA^{Ala} into both α - and β -chains (Fig. 3). On the other hand, although the β -chain of rabbit hemoglobin contains one cysteine residue,¹² no labeling of the β -chain was observed in the transfer

starting buffer described in the following section.

Chain separation: The globin was separated into its constituent α - and β -chains as described by Dintzis.⁸ The sample was applied to a 9 × 1 cm carboxymethyl cellulose (CMC) column (Serva Entwicklungslabor, Heidelberg, Germany, exchange capacity 0.54 meq per gram) and eluted using a linear gradient (150 ml 0.2 *M* formic acid, 0.02 *M* pyridine in the mixing chamber; 150 ml 2.0 *M* formic acid, 0.2 *M* pyridine in the reservoir). The separated α - and β -chains were precipitated with 5% trichloroacetic acid, washed with ethanol-ether 1:1, then with ether, and dissolved in water.

Isolation and analysis of the α -chain peptides: The α -chains were digested with trypsin and the peptides separated by paper electrophoresis and chromatography as described by Dintzis⁸ with the modification that β -mercaptoethanol, 5 μ moles per ml, was present during the entire procedure to keep the cysteine in the reduced form. The peptides were made visible by dipping the paper in 0.25% ninhydrin in acetone and identified by their positions.⁹ They were cut out, eluted with 0.1 *N* HCl, and counted in an end-window gas flow counter (Nuclear

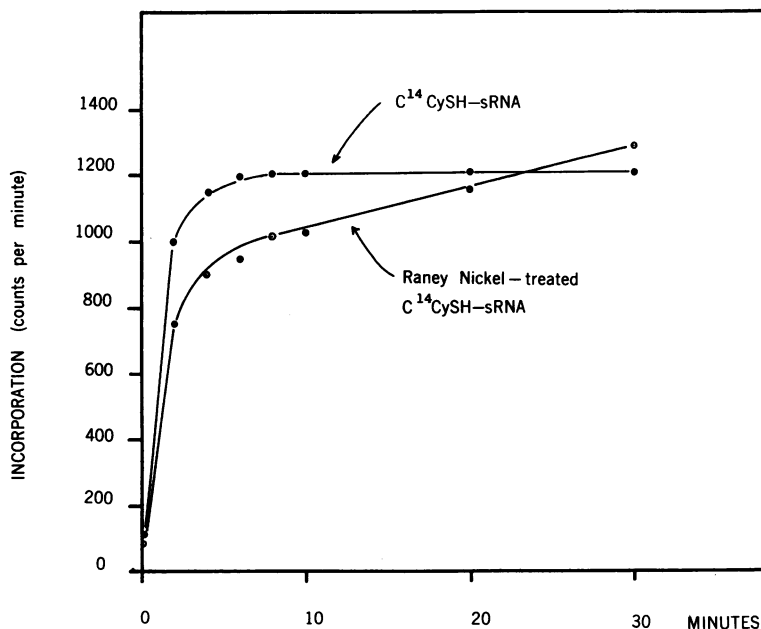


FIG. 2.—Kinetics of transfer of radioactivity into protein. Incubation conditions as stated in the legends to Figures 4 and 5. 0.1 ml aliquots of the total reaction mixture were precipitated, washed according to Siekevitz,¹⁷ and counted.

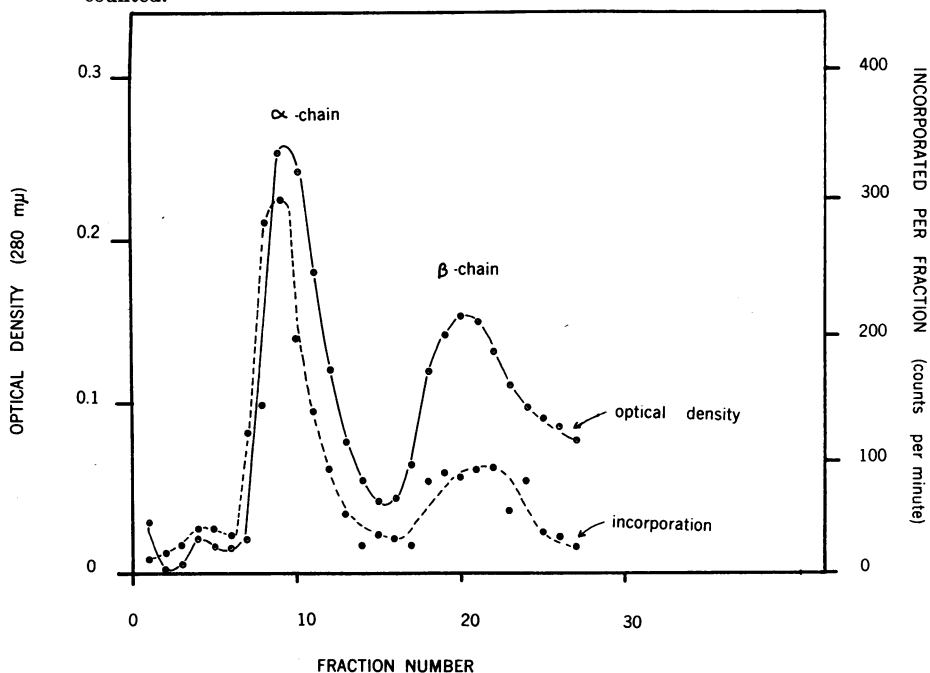


FIG. 3.—Separation of α - and β -chains of hemoglobin labeled in the rabbit reticulocyte ribosomal system by transfer from C^{14} Ala-sRNA^{Ala}. Conditions as described in *Methods*. 32 mg of ribosomes were incubated with 20 mg of *E. coli* sRNA charged with C^{14} alanine (1.35×10^5 cpm) and a complete mixture of nonradioactive amino acids minus alanine, in a final volume of 3.5 ml. Chromatography as described in *Methods*. The total volume of each fraction was 7 ml. 1 ml aliquots were dried on planchets and counted.

experiments involving either $C^{14}\text{CySH-sRNA}^{\text{CySH}}$ (Fig. 4) or Raney nickel-treated $C^{14}\text{CySH-sRNA}^{\text{CySH}}$ (Fig. 5). The reason for this is unknown. In any case, only the α -chain was used in the further analysis.

Note in Figures 3, 4, and 5 that the α -chain radioactivity is displaced to the left

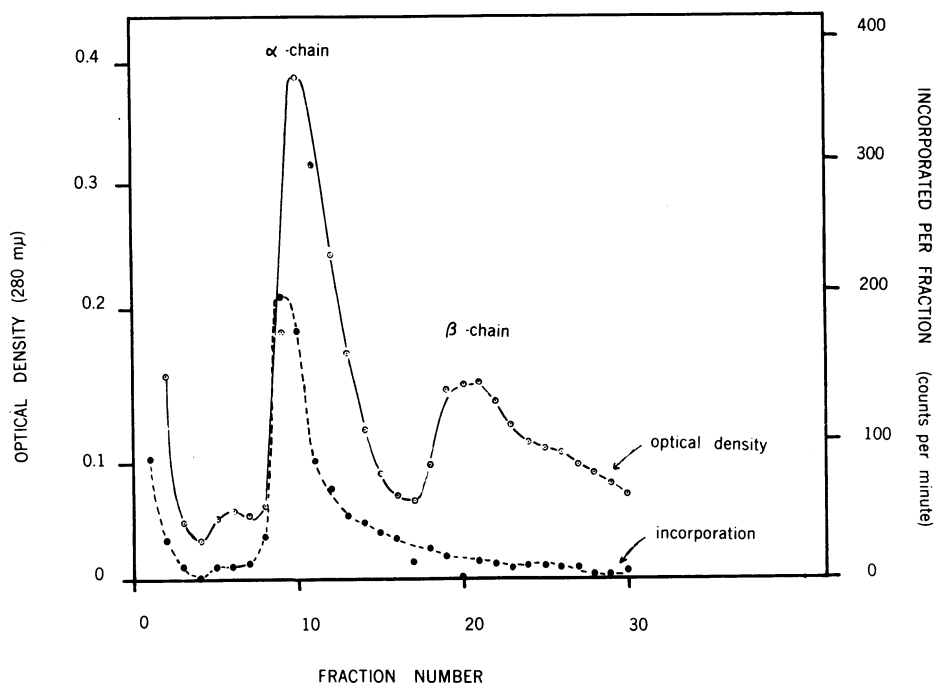


FIG. 4.—Separation of α - and β -chains of hemoglobin labeled in the rabbit reticulocyte ribosomal system by transfer from $C^{14}\text{CySH-sRNA}^{\text{CySH}}$. Conditions as described in *Methods*; 100 mg of reticulocyte ribosomes were incubated, in a final volume of 10 ml, with 20 mg of *E. coli* sRNA charged with C^{14} cysteine (63,250 cpm) plus 20 mg of C^{12} aminoacyl-sRNA charged with a complete mixture of nonradioactive amino acids minus cysteine. Chromatography as described in *Methods*. The total volume of each fraction was 7 ml. Aliquots of 2 ml were dried and counted.

relative to the optical density of the carrier, suggesting some abnormality of the labeled protein. However, a similar displacement is observed if the amino acids are transferred from rabbit liver sRNA,¹⁴ so that this effect cannot be ascribed to the use of *E. coli* sRNA in these experiments. When the hemoglobin is purified by chromatography on carboxymethyl cellulose prior to chain separation, no such displacement of the α -chain is observed.¹⁵

Distribution of radioactivity in α -chain peptides: The α -chains were digested as described and the individual peptides eluted and counted (see *Methods*). Table 1 shows the pattern of incorporation of labeled amino acids into various peptides as a function of the labeled aminoacyl-sRNA used.

According to Diamond and Braunitzer,¹¹ alanine should occur in peptides 1, 4, 9, 8 and 9, 10, 14, and 15. (The sequence: peptide 7-Lys-peptide 9- is hydrolyzed in two ways, yielding the fragments: peptide 7-Lys- plus peptide 9- or else peptide 7- plus Lys-peptide 9-. In the first case, peptide 7-Lys- is further split to yield free Lys-, designated as peptide 8. In the second case, however, Lys-peptide 9-

resists further hydrolysis and is designated "peptide 8 + 9.") In the hemoglobin made from C^{14} Ala-sRNA^{Ala}, the observed pattern of labeling is in agreement with that expected, except for peptides 1 and 4 which occur near the free-amino end of the chain. This is presumably due to the fact that the cell-free system does not start new chains, so that the observed labeling is derived from the completion of partly preformed chains.^{13, 16}

The cysteine-containing peptide number 13, obtained by chymotryptic digestion of the tryptic core, is the one of special interest here. No C^{14} alanine was found in this peptide after transfer of either C^{14} Ala-sRNA^{Ala} or C^{14} CySH-sRNA^{CySH}. However, using Raney nickel-treated C^{14} CySH-sRNA^{CySH} that contained 50 per cent of its radioactivity in the form of the hybrid C^{14} Ala-sRNA^{CySH}, hydrolysis of peptide 13 showed approximately 60 per cent of the counts to be alanine. By contrast, no detectable counts were transferred from the hybrid into any of the peptides that normally contain alanine.

It must therefore be concluded that the sRNA dictates the position of its attached amino acid in hemoglobin synthesis, corroborating the result obtained by Chapeville *et al.*³ with poly UG.

Summary.—"Rabbit" hemoglobin was prepared in a cell-free system using rabbit reticulocyte ribosomes and C^{14} alanine attached to the *E. coli* sRNA that normally carries cysteine. Alanine was incorporated into a peptide of the α -chain that normally contains cysteine but not alanine. Thus, in confirmation of the result previously obtained with a synthetic polynucleotide, the sRNA molecule acts as a specific adaptor in true protein synthesis.

The early stages of this work were carried out with the collaboration of Dr. William J. Ray, Jr., whose brilliant suggestion for preparing the Ala-sRNA^{CySH} hybrid by the use of Raney nickel made these experiments possible. We also wish to thank

TABLE 1
ANALYSIS OF ALANINE- AND CYSTEINE-CONTAINING PEPTIDES OF HEMOGLOBIN LABELED BY TRANSFER FROM VARIOUS AMINOACYL-SRNA'S

Peptide	No. of Residues Ala Cys	Hemoglobin Made from C^{14} Ala-sRNA ^{Ala}		Hemoglobin Made from C^{14} CySH-sRNA ^{CySH}		Hemoglobin Made from Raney Nickel-Treated C^{14} CySH-sRNA ^{CySH}	
		Total incorporation (cpm)	Analysis Ala Cys (cpm)	Total incorporation (cpm)	Analysis Ala Cys (cpm)	Total incorporation (cpm)	Analysis Ala Cys (cpm)
1	0	0.0	0.0	-0.1	0.2	-0.2	0.0
4	2	-0.3	-0.3	-0.1	-0.2	0.1	-0.2
9	0	1.4	0.9	0.0	0.2	0.1	0.1
8 + 9	1	2.9	1.3	0.2	-0.1	-0.4	0.0
10	3	9.1	4.7	0.1	0.2	-0.1	0.0
13	0	-0.2	-0.2	13.2	0.0	-0.1	0.2
14	1	4.0	2.2	0.2	0.0	10.2	3.1
15	3	15.3	7.6	0.1	0.1	-0.1	-0.1
						-0.3	-0.2

The peptides are numbered according to Diamond and Brautner¹¹ in order of their sequence in the polypeptide chain, starting from the free amino end. Peptide 13 is the one of special interest. Isolation and counting of the peptides is described in the text. In analysis of each peptide for its alanine and cysteine content, only about half the counts were recovered. Samples were counted for at least 300 counts.

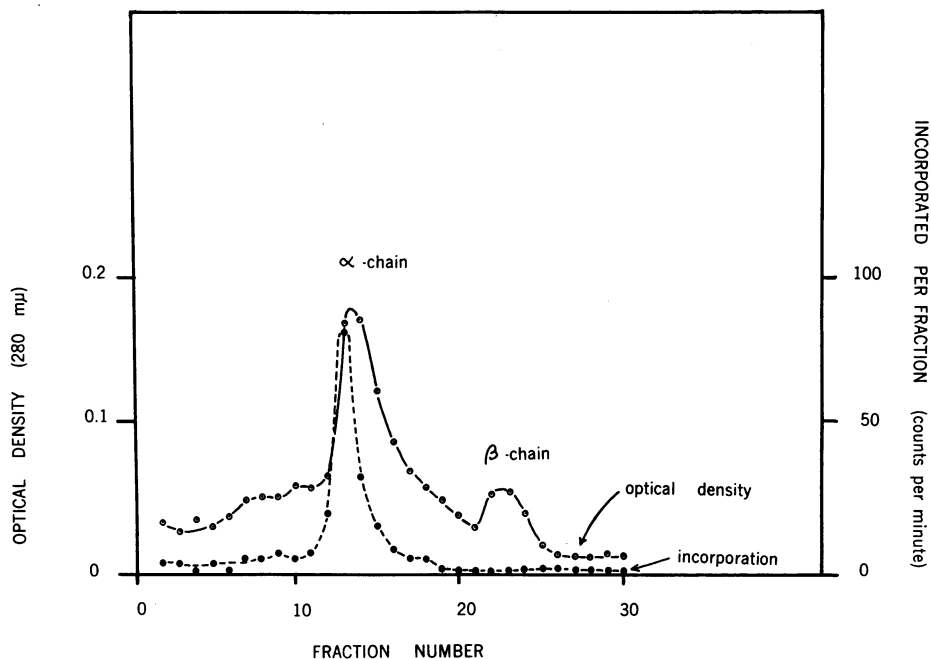


FIG. 5.—Separation of α - and β -chains of hemoglobin labeled in the rabbit reticulocyte ribosomal system by transfer from C^{14} CySH-sRNA^{CySH} treated with Raney nickel and containing 50% of its radioactivity as alanine and 50% as cysteine. Conditions as described in *Methods*. 100 mg of reticulocyte ribosomes were incubated, in a final volume of 10 ml, with 19.5 mg (59,500 cpm) of Raney nickel-treated *E. coli* sRNA (charged originally with C^{14} cysteine in the presence of C^{12} alanine) plus 20 mg of C^{12} aminoacyl-sRNA (cysteine omitted). Chromatography as described in *Methods*. The total volume of each fraction was 7 ml. Aliquots of 0.5 ml were dried and counted.

Dr. François Chapeville for a gift of C^{14} CySH-sRNA that was very helpful in the preliminary work. The excellent technical assistance of Mrs. Dorothy Dais is gratefully acknowledged.

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* sRNA = soluble ribonucleic acid, sRNA^{CySH} denotes the specific sRNA acceptor that combines with C^{14} cysteine to form C^{14} CySH-sRNA^{CySH}, poly UG = polyuridylic-guanilyc acid, PEP = phosphoenolpyruvate, GSH = reduced glutathione, GTP = guanosine triphosphate, ATP = adenosine triphosphate, Tris = trishydroxymethylaminomethane.

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PHOTOELECTRIC SPECTROPHOTOMETRY OF PLANETARY NEBULAE

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In 1953¹ we measured the relative intensities of the strongest lines in the spectra of the brighter planetary nebulae with the 24–36 inch Curtis Schmidt telescope at the University of Michigan. With the small light-gathering power of this telescope it was practical to observe only the green nebular lines of [OIII] and H β . An objective prism produced the spectrum which was then measured with a conventional photometer. Thanks to Director Bowen and the staff of the Mt. Wilson and Palomar Observatories, we were able to extend this study to other lines and to fainter planetary nebulae in 1956 by using the 60-inch and 100-inch reflectors at the Mt. Wilson Observatory.

For this latter investigation we employed a direct recording photoelectric spectrophotometer designed and built by Liller² with the aid of a grant from the University of Michigan's Rackham School of Graduate Studies. The scanner employs identical $f/5$ collimating and focussing systems. The spectrum, produced by a 600 groove/mm grating, has a dispersion of 64 Å/mm in the second order. As a red and infrared detector, we used a Farnsworth 16-PM-1 photomultiplier with a Ag-Cs₂O-Cs cathode. We used an EMI 6685 photomultiplier as our blue sensitive cell for these 1956 observations. Both cells were refrigerated. Slots and apertures of various sizes enabled us to accommodate nebulae of different angular diameters and to control spectral purity. We scanned the spectrum by rotating the grating at any one of 3 possible speeds: 30 Å/mm, 90 Å/mm, or 270 Å/mm.

Observations of suitable standard stars of presumably known energy distribution³ at both low and high altitudes in the sky permitted nightly determinations of the atmospheric extinction, and the response of the telescope-plus-spectrometer, both wavelength dependent quantities. Our basic standard star was Vega, although α Andromedae was occasionally used as a secondary standard.

Altogether, 30 nebulae were comprised in our program, but we give data here for 25 having the most extensive records. One diffuse nebula NGC 604 in M33 will be discussed separately. The Network nebula in Cygnus and several planetaries were so faint as to yield data on only the strongest lines.

Table 1 lists the nebulae in order of right ascension. The second column gives